Biomarkers of brain injury following an American football game

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Introduction

Concussion injury is defined as a complex pathophysiological process affecting the brain, induced by traumatic biomechanical forces[1], and is the most common head injury experienced by American football players.[2] However, head impacts commonly occur during American football games in which visible signs or symptoms of neurological dysfunction may not develop[3-6], despite those impacts having the potential for neurological injury.[7,8] Studies have shown that even a minor head injury with no concussive symptoms (subconcussion) can lead to abnormal results in neuropsychological tests, abnormal functional MRI (fMRI), and chronic traumatic encephalopathy (CTE).[7-11]

This raises the question of whether or not ordinary American football play, without incidence of concussion, may cause immediate brain injury. Therefore, the purpose of the current study was to determine if serum concentrations of biochemical markers of brain injury are elevated following an American football game in athletes who sustained subconcussive head impacts not significant enough to cause clinical symptoms of brain injury. Numerous biomarkers of head injury have been discovered and their efficacy of detecting and predicting severity of traumatic brain injury have been tested in the clinical setting.[12-14] However, few of these discovered biomarkers have been analyzed in their ability to detect sports-related head injury.[15] Since S100B and NSE have been monitored

quite frequently and appear to correlate well with number of head impacts they were chosen as the biomarkers for this study.[16-19] Creatine kinase (CK) and Cortisol serum levels were also measured to estimate muscle damage and stress respectively. It was hypothesized that NSE, S100B, and CK would increase significantly as a result of the football game, while cortisol levels would not increase significantly.

Materials and methods

Subjects

Seventeen (4 offensive lineman, 3 defensive linemen, 3 running backs, 3 linebackers, 2 quarterbacks, 1 defensive back, and 1 punter) male Division III collegiate American football players who were members of the junior varsity team participated in this study. Players had a mean age of 19. 5 years (SD = 0.94), height of 1.83 m (SD = 0.07), and lean mass of 70.6 kg (SD = 10.0). Informed written consent was obtained from all subjects and the University of Wisconsin-Platteville Institutional Review Board for the protection of human subjects (Platteville, Wisconsin) approved the study (IRB Protocol #2015-16-15).

Study design

Prior to the first and only junior varsity football game of the season (eight weeks following the start of the varsity season) anthropometric data was collected from all subjects. Body weight and height were measured using a physician scale and height rod (DETECTO,

Webb City, MO USA), while Dual-energy X-ray Absorptiometry (DXA) whole body scans were performed on all subjects using a Hologic Horizon Wi bone densitometer (Hologic, Inc., Marlborough, MA USA) to obtain lean mass measures.

Two days prior and approximately one hour following the junior varsity football game, venous blood was collected from all subjects. Pregame blood was drawn and each subject acted as his own control. Blood was drawn from a prominent vein in the antecubital space using a 21-gauge butterfly needle with a 7 inch luer lock extension (Becton-Dickinson, Franklin Lakes, NJ USA) connected to a vacutainer adapter (Becton-Dickinson, Franklin Lakes, NJ USA). The blood was collected into a 4 ml serum separation vacutainer tube (Becton-Dickinson, Franklin Lakes, NJ USA).

Biochemical analysis

Following blood collection all samples were allowed to incubate at room temperature for one hour. Immediately after the one hour incubation period each sample was centrifuged at 3500 RPM for 10 minutes at 4°C using a Thermo IEC Centra CL3 centrifuge (Thermo Fisher Scientific, Inc., Walthum, MA USA). Serum was then removed from each sample and placed in serum transfer tubes for subsequent analysis of NSE, S100B, and CK. Samples were then stored at 5°C for cortisol analysis which occurred within 72 hours of initial storage.

Neuronal specific enolase. NSE was measured using a commercially available enzyme immunometric assay (EIA) kit (CanAg NSE EIA 420-85, Fujirebio Diagnostics, Inc., Göteborg, Sweden). This is a solid phase, non-competitive immune assay utilizing two monoclonal antibodies (MAb) derived from mice. The monoclonal antibodies are specific for the γ-subunit of NSE and therefore detect both the γγ and αγ isoenzymes of NSE. The serum samples and calibrators were first added to streptavidin coated microstrips and then were incubated with both a biotinylated anti-NSE MAb and an anti-NSE MAb labelled with horseradish peroxidase (HRP). Following this incubation period a hydrogen peroxide (H_2O_2) and 3, 3', 5, 5' tetramethyl-benzidine (TMB) substrate solution was added. The HRP-H_2O_2 oxidizes the TMB forming a blue color in which the absorbance of the color at 620 nm is directly proportional to the concentration of NSE. All absorbance measurements were made using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

The known standards were then used to develop a calibration curve which was used to determine the NSE concentration in each serum sample. The sensitivity of the NSE assay ranged from 1-150 $\mu g \cdot L^{-1}$. Analysis of standards and samples were all performed in duplicate. The NSE assay performed before the football game gave a calibration curve with an $R^2 = 0.9977$ and the intra-assay coefficient of variation (CV) was 4.3%. After the

football game the NSE assay calibration curve was generated with an $R^2 = 0.9996$ and the intra-assay CV was 9.4%. Inter-assay CV between the pregame and post-game NSE assays was 6.3%.

Protein S-100B. Protein S-100B was measured using a commercially available EIA kit (CanAg S100 EIA 708-85, Fujirebio Diagnostics, Inc., Göteborg, Sweden). This EIA is a solid-phase, two-step non-competitive immunoassay utilizing two MAb from mice that are specific to the B monomer of protein S-100 such that the assay identifies both protein S-100BB and protein S-100A1B. The assay requires that the serum samples and calibrators are incubated in streptavidin coated strips with a biotinylated Anti-S100B MAb. Following this incubation period an Anti-S100B MAb conjugated with HRP is added to the strips and allowed to incubate before adding an H_2O_2 buffered TMB substrate. HRP-H_2O_2 then metabolizes the TMB forming a blue color at which the intensity is directly proportional to serum S100B concentration. The absorbance of this blue color was measured at 620 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The known standards were then used to develop a calibration curve which was used to determine the S100B concentration in each serum sample. The sensitivity of the S100B assay ranged from 10-3500 $ng \cdot L^{-1}$. Analysis of standards and samples were all performed in duplicate. The S100B assay performed before the football game gave a calibration curve

with an R^2 = 0.9942 and the intra-assay CV was 3.6%. After the football game the S100B assay calibration curve was generated with an R^2 = 0.9936 and the intra-assay CV was 4.9 %. Inter-assay CV between the pregame and post-game S100B assays was 5.2%.

Creatine kinase. Creatine Kinase was measured using a commercially available CK activity assay kit (Catalog No. MAK116; Sigma-Aldrich, St. Louis, MO, USA). CK is a dimeric protein made up of both muscle (M) and brain (B) subunits; however, this assay does not distinguish between types of CK. All serum samples and a calibrator were incubated with adenosine diphosphate (ADP), phosphocreatine (PC), hexokinase, glucose, glucose-6-phosphate dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADP). The CK present in each sample converts ADP and PC to ATP and creatine. The ATP is then used by hexokinase to phosphorylate glucose to glucose-6-phosphate. Glucose-6-phosphate is then oxidized by glucose-6-phosphate dehydrogenase forming NADPH and 6-phospho-D-gluconate. The production of NADPH was measured at 340 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The increase of absorbance at 340 nm is directly proportional to the activity of CK. One unit of CK is the amount of enzyme that will transfer 1µmole of phosphate from PC to ADP per minute at a pH of 6.0. This assay was carried out over a period of 20 minutes where the absorbance was read immediately after plate preparation (initial) and then after a 20 minute

incubation period (final). The following formula was used to calculate CK activity in Units·L^{-1}.

$$CK = \frac{(A_{340})_{final} - (A_{340})_{initial}}{(A_{340})_{calibrator} - (A_{340})_{blank}} \times 150$$

Where $(A_{340})_{final}$ = the final absorbance of NADPH at 340 nm, $(A_{340})_{initial}$ = the initial absorbance of NADPH at 340 nm, $(A_{340})_{calibrator}$ = the absorbance of the calibrator at 340 nm, $(A_{340})_{blank}$ = the absorbance of the blank (deionized water) at 340 nm, and 150 = the equivalent activity (Units·L^{-1}) of the calibrator when the assay is read at 20 minutes and 40 minutes. The sensitivity of the CK assay ranged from 30-1800 U·L^{-1}. The intra-assay CV was 3.8% before the football game. After the football game the intra-assay CV was 1.6%. Inter-assay CV between the pregame and post-game S100B assays was 3.6%.

Cortisol. Cortisol was measured using a commercially available enzyme-linked immunsorbent (ELISA) assay kit (Catalog No. SE120037; Sigma-Aldrich, St. Louis, MO, USA). This ELISA is a solid-phase, competitive immunoassay utilizing two anti-cortisol MAb. Serum samples and standards were incubated in anti-cortisol MAb coated wells with anti-cortisol MAb enzyme conjugate. TMB substrate was then added to the wells and the intensity of color is inversely proportional to cortisol concentration. The intensity of color

was measured at an absorbance of 450 nm using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Analysis of standards and samples were all performed in duplicate. The sensitivity of the cortisol assay ranged from 0-800 $ng \cdot ml^{-1}$. The intra-assay CV was 4.7%. Since the cortisol assay for both pre- and postgame samples were performed on the same plate at the same time there was no inter-assay CV. The cortisol standard curve is not linear; therefore, neither an R^2 value nor a predictive equation could be generated. Because of this, the cortisol concentrations were estimated visually based off the standard curve.

Statistical analysis

All statistical analyses were generated using SPSS version 22 (SPSS Inc., Chicago, IL USA). Means of NSE, S100B, CK, and cortisol serum concentration pregame were compared to post-game using two-tailed paired-samples t-tests for parametric data. Normality was analyzed using a Shapiro-Wilk test with an alpha level of 0.05, while homogeneity of variance was analyzed using Levene's test with an alpha level of 0.05. A Wilcoxon rank-sum test was used for data not meeting the assumption for normality, and a Welch t-test was used for data not meeting the assumption for homogeneity of variance. A 95% confidence interval (CI) was used to assign bounds of expected discrepancy between the sample mean and the population mean. A Cohen's d (d) test using the original standard

deviations (as opposed to the pooled standard deviations)[20] was performed to determine the effect size that the football game had on each variable. A two-tailed Pearson Correlation Coefficient was used to measure the relationship between S100B and body mass, along with S100B and CK. Data is reported as mean (SD) and alpha was set at 0.05 to determine statistical differences between means.

Results

Mean serum NSE concentration was significantly different between pregame and postgame ($p < 0.001$; d = 1.2; observed power = 0.915). Pregame serum NSE concentration was an average of 7.0 $\mu g \cdot L^{-1}$ (SD = 2.2; 95% CI: 5.8 – 8.1 $\mu g \cdot L^{-1}$) while mean serum NSE postgame was 13.1 $\mu g \cdot L^{-1}$ (SD = 7.0; 95% CI: 9.5 – 16.7 $\mu g \cdot L^{-1}$). Figure 1 shows mean ± SD NSE pregame and postgame data. The average serum concentration of S100B was also significantly greater postgame as compared to pregame ($p < 0.001$; d = 2.1; observed power = 1.000). Pregame serum S100B concentration was an average of 0.013 $\mu g \cdot L^{-1}$ (SD = 0.012; 95% CI: 0.007 – 0.019 $\mu g \cdot L^{-1}$) while mean serum S100B postgame concentration was 0.069 $\mu g \cdot L^{-1}$ (SD = 0.036; 95% CI: 0.050 – 0.087 $\mu g \cdot L^{-1}$). Figure 2 shows mean ± SD S100B pregame and postgame data.

Neither serum CK ($p = 0.116$; d = 0.56; observed power = 0.349) nor serum cortisol concentration ($p = 0.876$; d = 0.12; observed power = 0.059) changed significantly from

pregame to post game. Pregame serum CK concentration was an average of 90.5 $U \cdot ml^{-1}$ (SD = 41.9; 95% CI: 70.0 – 112.1 $U \cdot ml^{-1}$) while mean serum CK postgame concentration was 120.2 $U \cdot ml^{-1}$ (SD = 62.7; 95% CI: 87.9 – 152.4 $U \cdot ml^{-1}$). Figure 3 shows mean ± SD CK pregame and postgame. Pregame serum cortisol concentration was an average of 369.2 $nmoles \cdot L^{-1}$ (nM) (SD = 159.8; 95% CI: 287.0 – 451.4 nM) while mean serum cortisol postgame concentration was 353.0 nM (SD = 170.5; 95% CI: 265.3 – 440.6 nM) Figure 4 shows mean ± SD cortisol pregame and postgame data. Figure 5 shows that little correlation was found between postgame S100B serum concentration and body mass (R^2 = 0.029, p = 0.911); and low correlation was found between postgame S100B serum concentration and postgame CK serum concentration (R^2 = 0.353, p = 0.165).

[Insert figures 1-5].

Discussion

This research was conducted as a pilot study to determine if head injury occurs during one American football game due to subconcussive impacts without the occurrence of concussion or mild traumatic brain injury symptoms. During the football game no concussions or traumatic head injuries were reported by the players or observed by the athletic training staff. Despite no clinical evidence of brain injury both serum NSE and S100B increased significantly following the football game. The increase in serum levels of

NSE and S100B following the football game coincide with previous studies analyzing these two biomarkers before and after athletic competition.[16,17,21-26] Surprisingly, CK levels did not rise significantly following the football game as has been reported in past studies following athletic competition.[16,17,26,27] Cortisol has been shown to increase following head injury[28], including boxing bouts with punches to the head[16] but has not been reported to increase significantly following an American football game[27] when head injury is not present. The findings of cortisol not increasing following a sporting event in which no head injuries have occurred agrees with the results of our study.

The postgame serum concentration of 13.1 $\mu g \cdot L^{-1}$ for NSE is around the normal serum range of 12.5 $\mu g \cdot L^{-1}$ for healthy humans[14] and similar to those levels found following a game or match in other contact sports.[23–25] As expected, the concentration of 13.1 $\mu g \cdot L^{-1}$ following the football game was lower than that found in individuals following a boxing match (31.1 $\mu g \cdot L^{-1}$)[16] or karate event (20.2 $\mu g \cdot L^{-1}$)[17] in which punches and kicks to the head were prevalent. Interestingly, the median NSE concentration of 11.2 $\mu g \cdot L^{-1}$ found after the football game in our study was greater than the median concentration of 6.5 $\mu g \cdot L^{-1}$ found after concussion injury in ice hockey players.[22] This low level of NSE concentration in the serum of concussed ice hockey players has brought the usefulness of NSE as a marker of sports related head injury into question.[22]

The presence of NSE in thrombocytes and erythrocytes[14] also brings the usefulness of measuring NSE to assess head injury into question. There is concern of erythrocyte lysis during competition[29] and analysis[30] which may cause a false interpretation of elevated NSE. Indeed, a limitation to our study is that we did not measure hemolysis of blood samples. However, due to immediate processing and analyzing of the serum samples, and the normal serum concentrations of NSE observed in the pregame samples, it is unlikely that significant hemolysis due to our handling of the blood specimens occurred.[30] In addition, Skogseid et al. (1992) has reported that serum NSE levels in patients with multiple extracerebral injury have still been shown to correlate only with brain injury[31], implying that the brain is the main source of NSE. Further research into the usefulness of NSE as a biomarker of head injury caused by sports is warranted.

Postgame, the mean S100B serum concentration of 0.069 $\mu g \cdot L^{-1}$ was within the normal serum concentration of 0.2 $\mu g \cdot L^{-1}$ found in healthy humans.[14] This level was also lower than the values found post competition in swimmers[32], runners[18,33], soccer players[24,25], ice hockey players[23], basketball players[23], boxers[16,18], karate practitioners[17], and concussed ice hockey players[22]. However, the postgame serum concentration was greater than found in subjects following cycling exercise.[18] The change of serum S100B levels from pre to post game (0.056 $\mu g \cdot L^{-1}$) was similar to that found in other non-concussed football players.[21,34]

As with NSE, the usefulness of S100B as a biomarker for head injury in sports has been called into question[22,32,33] due to extra cranial sources of S100B in adipocytes, melanocytes, chondrocytes, and skeletal muscle tissue.[35]

Support for the idea that S100B is a marker of muscle damage and not glial damage comes from studies that have found similar increases in both CK and S100B following running exercise[26,33] along with increases in S100B after swimming.[32] There is also evidence that subjects with greater body mass have greater levels of serum S100B.[36] However, other studies have reported no increases in serum S100B in the absence of impacts to the brain[18, 26,37,38], and Pham et al. (2010) have reported that extracranial sources of S100B do not affect serum levels.[39] Our results support the idea of serum S100B not being affected by extracranial sources as S100B was not correlated with CK or body mass.

In Conclusion, the effect of the football game on levels of NSE (d = 1.2) and S100B (d = 2.1) were large. However, the low NSE and S100B levels in the serum of subjects following this football game likely indicate two different possibilities: 1. That American football may be no more dangerous than other sports that cause axial vibrations or direct impacts to the head or 2. NSE and S100B may not be the ideal biomarkers for assessing head injury.

Several limitations must be taken into consideration with this study. First of all, since this was a pilot study, there was a small number of subjects and lack of a separate control group doing no exercise, a group performing non-contact exercise, and a group that did not receive any playing time during the game. Furthermore, this data was collected from one game within a cohort of athletes who have not played any other games during the season. This was a junior varsity game in which many substitutions occurred to give the majority of players a chance to compete and prove their talent to the coach. In contrast, starting offensive and defensive varsity players participate in approximately 13 games per year and typically remain in the game for the full duration of offensive or defensive play, which may result in greater NSE, S100B, and CK serum concentrations in subjects following varsity games. Additional research must also be conducted in varsity athletes and national football league (NFL) players to determine if the findings of this study are similar when elite athletes with a greater body mass play the game of American football for a longer time period.

There may also be biomarker differences by position which can be alluded to in our results but must be studied with a greater number of subjects per position for statistical inference. The use of equipment to gain biomechanical data such as: number of head and body impacts, magnitude of impacts, and location of impacts would also be of valuable

assessment in conjunction with biomarker data. Lastly, although the results of this study indicate that American football does not inherently cause immediate brain injury, there is likely occurrence of brain injury with reported concussions, which may be detrimental to participation. Although the data presented here is interesting and unique, future studies should consider the use of larger subject numbers and a control group.

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest

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